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PARADOXICAL NATURE OF MINERALOCORTICOID RECEPTOR ANTAGONISM BY PROGESTINS

[+]M. K. Agarwal and [++]J. Paillard

[+]CNRS and [++]UER Broussais-Hôtel Dieu, Paris, France

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SUMMARY: Progesterone, but not the synthetic progestagen R-5020, was as good as aldosterone in displacing the mineralocorticoid from its specific receptor in rat kidney both in classical competition studies and in aldosterone binding to MR_1 and MR_2 components of MR during physical separation. Paradoxically, at equimolar (10^{-8} M) concentrations, both gestagens (progesterone = 1, R-5020 = 20) were preferentially bound to the MR_4 component which coeluted with serum bound ^{14}C-corticosterone (or ^{14}C-progesterone), which could not be labelled with aldosterone in the kidney, and which could not be detected in the liver and the serum under any condition. The MR_1 entity, at 10^{-8} M, was saturated as: aldosterone = 2, R-5020 = 1 = progesterone. Thus, the agonist and the antagonist function in mineralocorticoid action may be expressed by two or more distinct and different sites.

INTRODUCTION: In an initial step, corticosteroid hormones appear to bind to their target specific receptor with subsequent translocation of the complex to nuclear acceptor sites (1). 'Antihormones' may act either by reversing this sequence or via association with another cellular vector (2). Progesterone opposes aldosterone effects in patients with Addison's disease, inhibits aldosterone mediated natriuretic diuresis (3), and antagonises receptor-agoist binding in competition studies (4). Contrary to the hypothesis of a unitary vector, we have demonstrated polymorphism of receptor for mineralo- and gluco- corticoids during physical separation (5), and this seems true of the receptor for all five classes of steroid hormones (1). In the present study, an "ideal" synthetic progestin (6), R-5020 (promegestone 17-α--methyl), only recently synthesized in the tritiated form, was used to demonstrate that mineralocorticoid antagonism by an antihormone may proceed via saturation of that subpopulation in the kidney that does not bind aldosterone.

Abbreviations used are: MR = mineralocorticoid specific receptor; GR = glucocorticoid specific receptor.

[+]Address after 15 July 1979: 15 Rue de l'Ecole de Médecine, 75270 Paris Cedex 06, France, to whom all correspondance should be sent.

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MATERIALS AND METHODS: Male, Wistar rats (150-200 g) were bilaterally adrenalectomised 2-3 days prior to use; food and 1% NaCl were provided ad libitum after surgery. Animals were sacrificed under ether anaesthesia, the desired organ was perfused with the initial buffer, according to the experimental procedure, and cell sap was finally obtained by centrifugation at 105,000 g (50 min). Blood, obtained by aortic cannulation was allowed to clot at 37°C (30 min), then at 4°C (60-90 min), and finally centrifuged at 3000 g.

For competition and association studies, 0.5 ml cytosol (in 0.01 M Tris-HCl, pH 7.4), or serum, was incubated (60 min 4°C) in presence of a desired amount of the radioactive steroid alone or in presence of a known excess of the cold homologous or heterologous molecule. Free steroid was removed by the addition of 0.5 ml (50 mg/ml) of activated charcoal (Sigma C-5260), further incubation (10 min 4°C), and centrifugation (15 min, 4000 g). Aliquots of 0.5 ml were mixed with 10 ml Scintix (Isotec, France) and counted in a Packard Tricarb Scintillation Spectrometer (1,2,5). Protein was quantitated by the Biuret method and all optical densities were read manually.

For chromatography, organ cytosol, prepared in the initial buffer as per elution procedure, was equilibrated with the steroid and charcoal treated as above, and loaded onto the resin as described previously (1,2,5). Undiluted serum was similarly treated; further details are given in appropriate legends.

1,2,^{3}H-aldosterone (53 Ci/m mol; lot 32), 1,2,6,7,^{3}H-progesterone (95 Ci/m mol; lot 26), 4-^{14}C-corticosterone (52 mCi/ m mol; lot 12), and 4-^{14}C-progesterone (61 mCi/m mol; lot 38) were purchased from Amersham (U.K.). ^{3}H-R-5020 (promegestone 17-α-methyl; 87 Ci/m mol; lot 1009-149) was a product of New England Nuclear (USA). Radiochemical purity exceeded 98% in all cases.

RESULTS AND DISCUSSION: Data in Fig.1a show that the kinetics of binding of aldosterone was similar in both the heart and the kidney, the two known targets of mineralocorticoid hormones (1). However, progesterone binding appeared to exhibit somewhat different association characterstics in the heart vs the kidney. Serum aldosterone was bound proportional to the dose of the steroid; these mostly confirm earlier data in the literature (3,4).

Data in Fig.1b establish that far greater quantities of R-5020 could be bound to kidney cytosol, despite comparable association characterstics of aldosterone and the "ideal" progestin (Fig. 1a vs 1b). Furthermore, R-5020 binding to vectors in the kidney was far greater than in the liver, whereas diluted (1/10) serum exhibited no binding at all (Fig. 1b).

Data in Fig.1c confirm that progesterone was as good as aldosterone in displacing ^{3}H-aldosterone from its receptor, whereas R-5020 was far less effective in this regard, as reported for other synthetic gestagens (3,4). In a reciprocal experiment (not shown), cold aldosterone very poorly displaced kidney bound ^{3}H-R-5020. In view of receptor multiplicity (1,2,5), do these three steroids (aldosterone, progesterone, R-5020) bind the same vector?

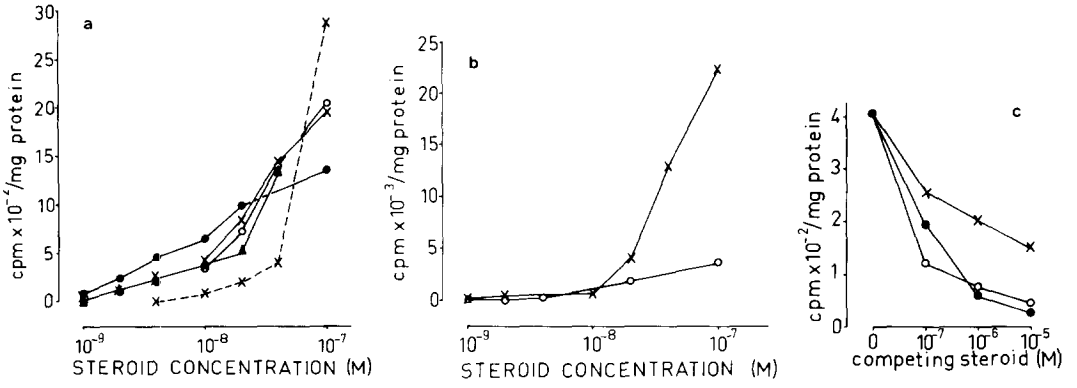

Fig. 1. ASSOCIATION CHARACTERSTICS OF STEROID BINDING IN SEVERAL RAT TISSUES

For Fig.1a, renal cytosol was equilibrated with ^{3}H-aldosterone (X) or ^{3}H-progesterone (▲), myocardial with either ^{3}H-aldosterone (○) or ^{3}H-progesterone (●), and serum with ^{3}H-aldosterone (X-----X). For Fig.1b, renal (X) or hepatic (○) cytosol was incubated with ^{3}H-R-5020. For Fig.1c, renal cytosol in presence of 10^{-8} M ^{3}H-aldosterone was put in contact with the indicated concentration of non-radioactive aldosterone (●), progesterone (○), or R-5020 (X). Non-specific binding in all cases was calculated by determination of radioactivity in presence of one thousandfold excess of cold, homologous steroid and was subtracted from the corresponding value obtained with tritiated hormone alone. Each sample (cold + radioactive or radioactive alone) was studied in triplicate at each point shown in the figure. For other details see Methods and earlier publications (1,2,5).

Results in Fig.2a further establish aldosterone (10^{-8} M) binding to MR_1, MR_2 and GR type of receptors (5); T = position of transcortin elution. Even 10^{-7} M progesterone did not label aldosterone-specific MR_2 whereas the labelling of MR_1 was greater than with aldosterone (Fig. 2a vs 2b). More importantly, huge quantities of the progestagen were bound to MR_4 which coeluted with serum transcortin (T) bound progesterone (Fig.2b). Similar results were obtained with myocardium cytosol (Fig.2c) where qunatitatively less progesterone was bound than in the kidney (please note the difference in scale). Since aldosterone binding to all components in Fig.2a diminished in presence of 100 fold higher unlabelled progesterone (confirming Fig.1c), antagonism of aldosterone action can proceed via displacement of the mineralocorticoid from its specific receptor in the region of progestagen excess (3,4); however, at equimolar (10^{-8} or 10^{-7} M) concentrations, in the physiological range, aldosterone saturates a specific MR_2 and progeserone the MR_4

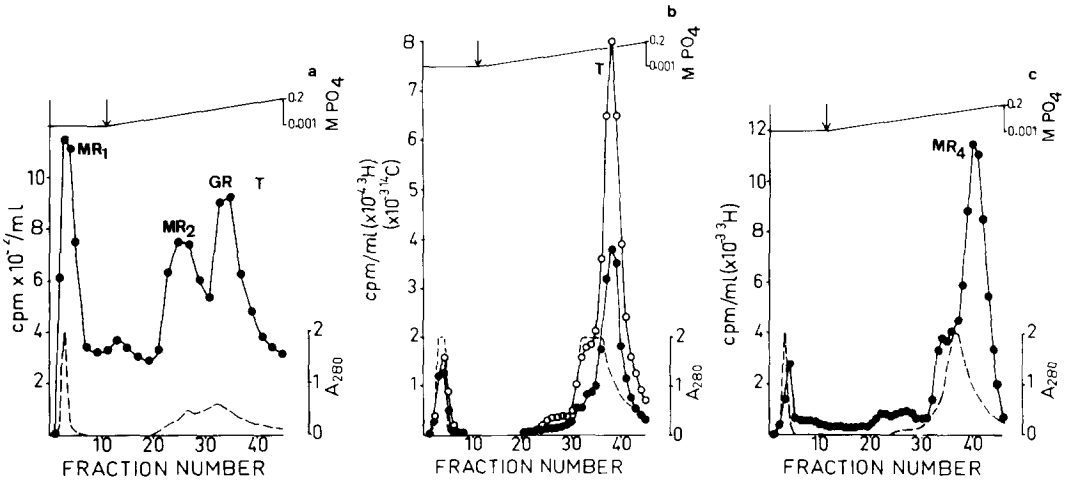

Fig. 2. ION EXCHANGE SEPARATION OF STEROID BINDERS ON DEAE-52 COLUMNS

5 ml renal cytosol was incubated with 10^{-8}M ^{3}H-aldosterone (a); 4 ml of either kidney (b) or myocardium (c) cytosol was incubated with 10^{-7}M ^{3}H-progesterone; for Fig.2b, 2 ml serum was incubated in presence of 0.5 µCi of ^{14}C-progesterone; all samples were charcoal treated (100 mg/ml mixture), separately, mixed for Fig.2b, and loaded onto DE-52 columns (1 x 25 cm). After a low ionic prewash (0.001 M phosphate, pH 7.5), elution was begun (at the arrow) with a gradient between 60 ml each of 0.001 M and 0.2 M of this buffer, pH 7.5. 1 ml samples were processed for radioactivity and the absorbance was recorded manually; further details in Methods and (1,2,5).

----------A_{280}; ●——● ^{3}H; ○——○ ^{14}C

which is not transcortin first because R-5020 does not label this carrier (see below) and, second, because R-5020 competes with progesterone for MR_4.

Five fold greater quantities of radioactivity eluted in the MR_4 region when R-5020 (10^{-7} M) was used in place of progesterone, but the order was reversed if MR_1 was considered (Fig. 3a vs 2b). The tremendous quantities of kidney bound R-5020 in the MR_4 region are to be contrasted with a small peak of radioactivity obtained when undiluted serum was used in place of renal cytosol (Fig.3b) and this was neither specific (abolished in presence of excess cold R-5020) nor coincided with serum bound ^{14}C-corticosterone which coelutes with MR_4 (Fig.3a vs 3b). Furthermore, liver bound R-5020 (Fig.3c), too, eluted in the same position as the serum-R-5020 complex (Fig.3b), was nonspecific (displaced by excess of cold R-5020), was 50 times less than MR_4

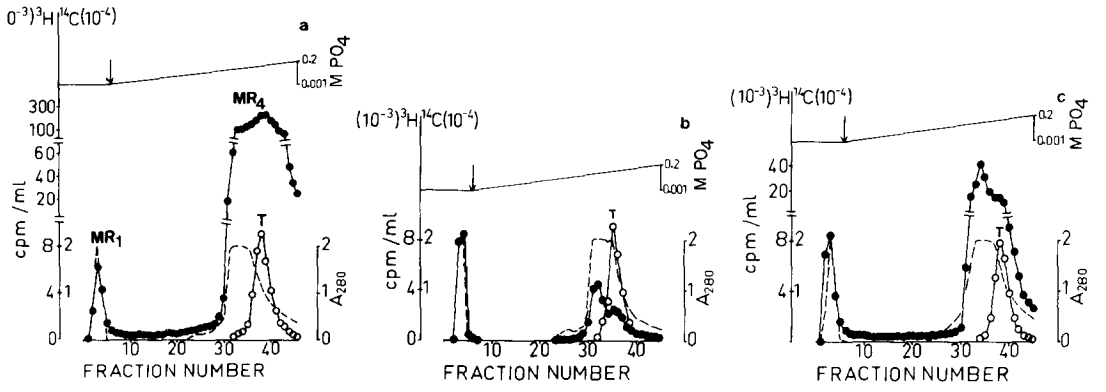

Fig. 3. ION EXCHANGE SEPARATION OF R-5020 BINDERS ON DEAE-CELLULOSE-52

4 ml renal cytosol (a), undiluted serum (b), or liver cytosol (c) was incubated with 10^{-7} M ^{3}H-R-5020; 2 ml fresh serum with 0.5 μCi ^{14}C-corticosterone was used for cochromatography as for Fig. 2 and (1,2,5).

----------A_{280}; ●——● ^{3}H; ○——○ ^{14}C

bound R-5020 in the kidney (Fig.3a vs 3c), and did not coelute with serum transcortin bound ^{14}C-corticosterone (T) where MR_4 is eluted. Thus, whereas R-5020 was less potent than progesterone in displacing aldosterone from its receptor in competition studies (Fig.1c), MR_4 (at 10^{-7} M) was saturated in the order: aldosterone = 0, progesterone = 1, R-5020 = 5 (at 10^{-8} M Progesterone = 1, R-5020 = 20, not shown), and this was totally wanting in the serum (Fig.3b) and the liver (Fig.3c) which is not a target for mineralocorticoids and is devoid of MR (5). Collectively, these data suggest progestagen action via saturation of MR_4, aldosterone via MR_2, and a common locus (MR_1) revealed (at 10^{-8} M) in the order aldosterone = 2, progesterone = 1 = R-5020).

Molecular filtration of steroid receptors revealed peaks A and B (122,000 and 67,000 daltons, respectively) of bound aldosterone in renal cytosol (Fig.4a) followed by a broad free peak (since untreated with charcoal). On the other hand, bound progesterone revealed an additional peak (C) in both renal (Fig.4b) and myocardial cytosols (Fig.4c) with another peak (D) evident only in the kidney. With R-5020 in place of progesterone, this peak (D) was 50 times more abundant, C was wanting, and A & B were comparable

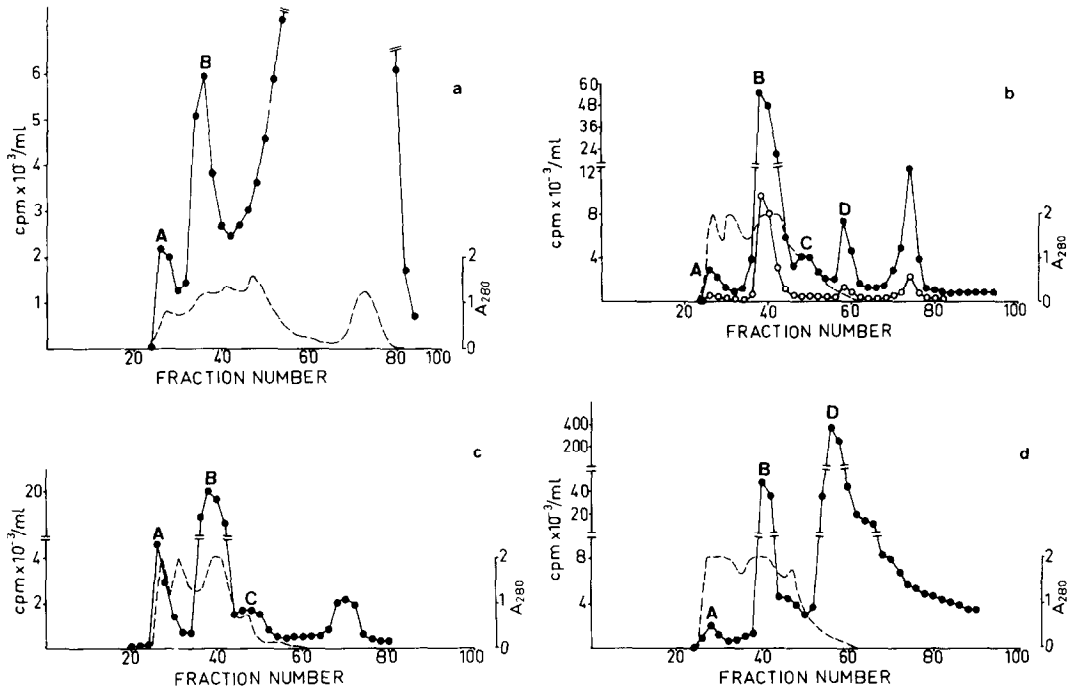

Fig. 4. MOLECULAR FILTRATION OF STEROID BINDERS ON ULTROGEL ACA-44 RESIN

2 ml renal cytosol was incubated with 10^{-7}M of either ^{3}H-aldosterone (a), ^{3}H-progesterone (b), or ^{3}H-R-5020 (d); 2 ml myocardium cytosol with 10^{-7} M progesterone was used for Fig.4c; for Fig.4b, 1 ml serum with 0.25 µCi of ^{14}C-corticosterone was utilised. Except for 4a, charcoal treatment was used in all cases for better resolution of peaks. Equilibration and elution were carried out with 0.01 M phosphate + 0.1 M NaCl on columns of 1 x 130 cm, as previously described in detail (1,2,5).

----------A_{280}; ●——● ^{3}H; ○——○ ^{14}C

(Fig.4d). The last peak in Figs.4b and 4c corresponds to radioactivity that dissociated during elution. Serum bound progesterone coeluted with peaks A and B (Fig. 4b) and would argue against polymerisation as the factor for the genesis of these peaks (C,D), as explained after rechromatography (1).

On Sephadex A-25 columns, based both upon molecular weights and charge, peak B was evident only with aldosterone-renal cytosol complex (Fig. 5a); peaks A, C, and D were seen with ^{3}H-progesterone in both kidney (Fig. 5b) and the heart (Fig.5c); serum bound ^{14}C-progesterone coeluted only with peak D (Fig.5b). Again, the relative abundance of the various components was a function of the steroid (aldosterone vs progesterone in the kidney) as well

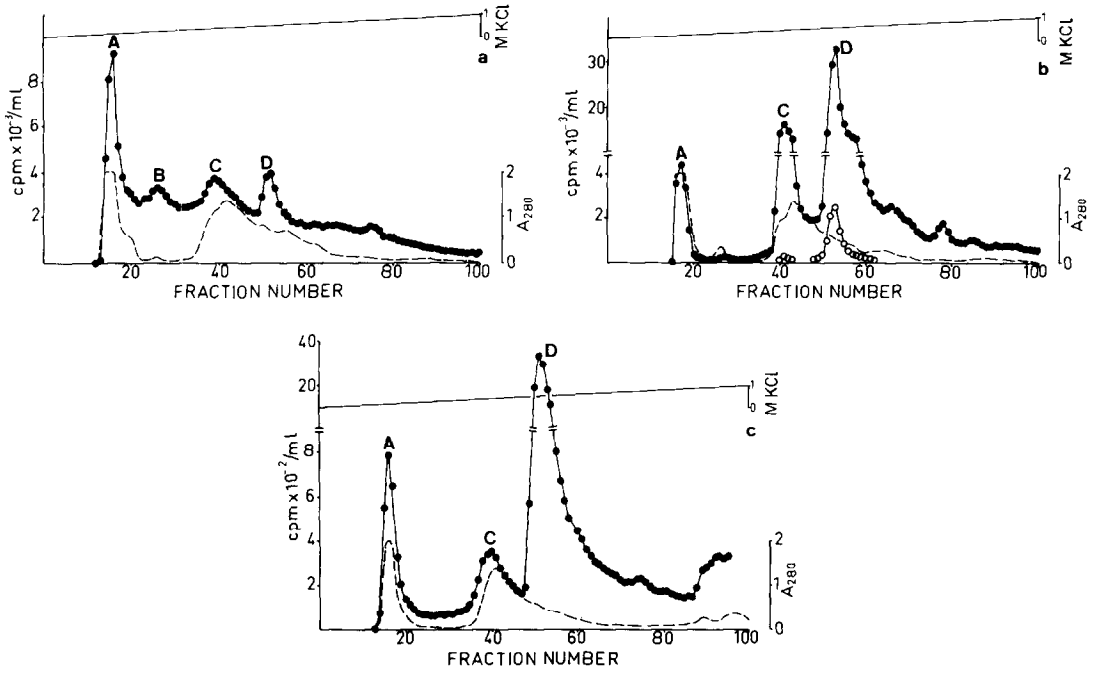

Fig. 5. SEPARATION OF STEROID BINDERS ON SEPHADEX A-25 COLUMNS

4 ml renal cytosol + 10^{-7}M of either ^{3}H-aldosterone (a) or ^{3}H-progesterone (b), or 4 ml cardiac cytosol + 10^{-7} M ^{3}H-progesterone (c) were charcoal treated prior to chromatography on 1.6 x 90 cm columns equilibrated with 0.01 M Tris-HCl, pH 7.5 and eluted with a gradient between 0 and 1 M KCl in this buffer. For Fig. 5b, 2 ml serm + 0.5 µCi ^{14}C-corticosterone was used (1,2,5).

----------A_{280}; ●——● ^{3}H; ○——○ ^{14}C

as the nature of the tissue (progesterone in the kidney vs the heart), as also evident with Ultrogel-ACA-44 chromatography above.

As before (3,4), competitive displacement of aldosterone from its specific receptor in renal cytosol was confirmed here with progesterone, but not R-5020 (an "ideal" gestagen) which has never been studied in this context. Indeed, huge quantities of R-5020 >>> progesterone were bound to MR_4 that was absent from non targets (liver, serum), and that was clearly distinct from renal MR_2 bound aldosterone, when physiological concentrations of these molecules were employed. Since deoxycorticosterone, a natural mineralocorticoid agonist, also eluted in the same position (previously denominated MR_3 in order of elution) in the kidney (7), but not the liver,

progestagens may possess weak mineralocorticoid activity (3); the transcortin-like MR_4 may be the equivalent of the unusual binder observed in kidney autoradiography (8). The discordance between competition studies vs physical separation establishes that mere Scatchard type of analyses are not adequate for the interpretation of receptor activity and may even be misleading. Although kidney may be endowed with high specific progesterone receptors, the possibility of receptor maturation from an uncommitted protein has been presented in our Multipolar model (1,2).

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